Pyrimidine Metabolism in Tissue Culture Cells Derived from Rat Hepatomas

I. Suspension Cell Cultures Derived from the Novikoff Hepatoma

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SUMMARY

Culture conditions are described for several cell lines derived from the Novikoff rat hepatoma. Stability of the stem cell line is demonstrated by a constant generation time of 10 hr for more than 360 transfer generations, and by its continued ability to produce typical animal tumors after 4 years in culture.

As in the parent tumor, no catabolism of thymine or uracil could be demonstrated. Complete inhibition of growth of the cultures by 10^{-4} M thymine and 10^{-4} M 5-fluoro-2'-deoxyuridine, and complete reversal of amethopterin and 5-fluoro-2'-deoxyuridine toxicity by 10^{-4} M thymidine, provide indirect evidence for high levels of thymidine kinase in these cells. Thymidine toxicity was shown to be readily reversed by 10^{-4} M deoxycytidine.

Two variant strains of cells are described, one of which apparently lacks thymidine kinase, since it is irreversibly inhibited by amethopterin in the presence of thymidine and is resistant to high levels of both thymidine and 5-fluoro-2'-deoxyuridine. The 2nd variant strain possesses thymidine kinase, but is resistant to high levels of thymidine.

Recent studies on chemically induced transplantable rat hepatomas have demonstrated that a spectrum of such tumors exists with varying degrees of change from the parent hepatocyte (26–30). Such hepatocarcinomas as the Morris 5123 (18) and Reuber H-35 (34), for instance, resemble the parent tissue to a marked degree both morphologically and biochemically (27), and thus fall in the category termed “minimal deviation hepatomas” by Potter (31). On the other hand, tumors such as the Novikoff hepatoma differ in so many respects from the parent tissue that one can only speculate as to their cell of origin.

In undertaking a program of comparative enzymology of such a diverse spectrum of tumors in the intact host, serious difficulties arise in terms of both experimental design and interpretation of data. Among these are the problem of distinguishing effects due to the intrinsic bio-

1 This work was supported in part by grant CA-00646 from the National Cancer Institute, NIH, USPHS. The data reported in this paper constitute a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree by P. A. Morse, Jr., University of Wisconsin, 1963.

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as the Morris 5123 and Reuber H-35, and hopefully, to include eventually cell lines derived from parenchymal liver itself.

The area of pyrimidine metabolism having been of interest in this laboratory for a number of years, a logical tumor with which to begin such a program was the Novikoff hepatoma, since this tumor apparently lacks the enzymes of thymine and uracil catabolism (29, 32). The purpose of this report is to describe the biologic properties and culture conditions of several cell lines derived from the Novikoff hepatoma, and to establish some of their biochemical characteristics, with particular reference to pyrimidine metabolism.

MATERIALS AND METHODS

All cell lines used were derived from a parent strain, (N1-S1), established from the ascitic fluid of a Novikoff hepatoma (23) by Dr. H. E. Swim (unpublished data) in the autumn of 1959 while on leave from Western Reserve University to McArdle Memorial Laboratory. This line of cells has been carried in suspension culture since shortly after its establishment, and had not been cloned prior to its receipt by one of us (P. A. M.). Periodic assays of tumor-forming ability during the 1st 6 months in culture were performed by injecting approximately 6 X 10^6 cells per animal i.p. into male Holtzman rats of 80-140 gm weight, obtained from the Holtzman Rat Co., Madison, Wis. Typical Novikoff tumors resulted with better than 90% incidence of take, with approximately 80% of the animals showing gross tumors in 10-20 days (H. C. Pitot, H. E. Swim and P. A. Morse, Jr., unpublished data).

The basal medium (designated 5-77) for all cultures was a modified Eagle's medium (5), lacking biotin, and fortified with supplementary amino acids and inositol (Table 1). The complete growth medium contained 5% whole beef serum, unless otherwise indicated, and 0.1% (w/v) Pluronic F-68 (Wyandotte Chemical Co., Wyandotte, Mich.) as described by Swim (22), to prevent denaturation of serum protein during agitation with resultant death of the cells. The basal medium was prepared and stored in bulk as a dry powder which had been ball-milled and desiccated (41). Such a procedure insures a high degree of uniformity with time in the final growth medium, and leads to greater ease of preparation as well. Glutamine, cystine, and penicillin were omitted from the powder for reasons of stability and solubility, and calcium chloride was omitted because of its hygroscopic properties. Cystine and calcium chloride were added at the time of preparation of a double strength stock basal medium; penicillin, serum, and Pluronic F-68 were added to the final growth medium just prior to filtration; glutamine was prepared as a separate sterile stock solution which was added either directly to culture flasks or to small amounts of medium just prior to use. The pH of the complete medium was adjusted to 7.6-7.8 with 5% CO_2 in air just prior to sterilization.

All media and experimental additives were sterilized before use by suction filtration through Selas 02 porosity filter candles or funnels, and routine sterility tests were conducted on blood agar base plates before each batch of medium was used. All media were used within 6-8 weeks after preparation.

Table 1: Composition of Basal Medium S-77

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
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<tbody>
<tr>
<td>Calcium chloride</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.6</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.8</td>
</tr>
<tr>
<td>Phenol red (sodium salt)</td>
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</tr>
<tr>
<td>Potassium chloride</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>116.2</td>
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<tr>
<td>Sodium bicarbonate</td>
<td>26.2</td>
</tr>
<tr>
<td>Monosodium phosphate</td>
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</tr>
<tr>
<td>L-Alanine</td>
<td>0.3</td>
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<tr>
<td>L-Aspartic acid</td>
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</tr>
<tr>
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<tr>
<td>L-Cystine</td>
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<td>L-Histidine</td>
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<tr>
<td>Glycine</td>
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<td>L-Isoleucine</td>
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<tr>
<td>L-Leucine</td>
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<tr>
<td>L-Lysoleucine</td>
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</tr>
<tr>
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<td>L-Phenylalanine</td>
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<tr>
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<td>L-Serine</td>
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<td>L-Tyrosine</td>
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<td>L-Valine</td>
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<tr>
<td>Choline bitartrate</td>
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<td>Folic acid</td>
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<tr>
<td>Calcium pantothenate</td>
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<tr>
<td>Mesoinositol</td>
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<tr>
<td>Nicotinamide</td>
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<tr>
<td>Pyridoxal HCl</td>
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<tr>
<td>Riboflavin</td>
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</tr>
<tr>
<td>Thiamin</td>
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</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>0.008</td>
</tr>
<tr>
<td>Penicillin G sodium</td>
<td>100 units/ml</td>
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</table>

Test compounds were prepared as sterile stock solutions in single strength basal medium, with concentrations adjusted where possible so that final additions to the growth medium were 1.0% or less by volume.

The beef serum used in the complete growth medium was prepared from blood collected at a local slaughterhouse from government inspected steers. Pooled blood from at least 8-10 animals was used, and the nonsterile serum was stored for up to 2 years at -20°C. Occasionally, commercial serum was used, but thus far no differences in growth rates or biochemical properties of the cultures have been detected due to differences in the source or time of storage of the sera.

Cell cultures were grown as suspensions in silicone-coated Florence flask of either 125 or 500 ml capacity, holding respectively 90 ml or 350 ml maximum volume of fluid. These flasks were altered; the normal necks were replaced with equal length necks fabricated from the tops of 25 by 150 mm screw cap Pyrex test tubes. Rubber-lined bakelite caps were used as closures, providing a much more convenient container than conventional rubber-stoppered flasks.

Silicone coating of the flasks was accomplished by
wetting the interiors with a clear solution obtained by suspending 2 oz of Dow-Corning silicone stopcock grease in 500 ml of carbon tetrachloride, settling for 48 hr and decanting the supernatant. After draining the flasks, the silicone was baked on at 135°C for 4—5 hr. Flasks were then washed as usual.

Cell suspensions were incubated at 37°C ± 0.5°C in a New Brunswick Gyrotory shaking incubator (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) operating at approximately 180 strokes/min. The shaking rate was found not to be critical to within at least −50% to +100%.

Cell counts were accomplished using the citric acid-crystal violet technic of Sanford et al. (36) as simplified by Swim and Parker (38, 40). Aliquots of 8, 6, 4, or 2 ml of cell suspension, as appropriate, were centrifuged and resuspended in 2 ml of 0.3 m citric acid containing 0.05% crystal violet. Tubes were shaken 3—4 min by hand and then counted in a Neubauer bright-line hemacytometer either immediately or stored at 4°C for counting at a later date. No significant differences in counts of duplicate samples stored up to 4 weeks were observed, although in all experiments counts were made within 3 weeks of the time of sampling. All cell counts were performed at least in quadruplicate for each given sample or time point.

Cell stocks were subcultured on alternate days by diluting appropriate aliquots to approximately 5 × 10^4 cells/ml. All cells used for growth experiments were harvested from cultures in logarithmic growth (approximately 5 × 10^4 cells/ml). Whenever possible, experimental cultures were prepared by direct dilution of stock cell suspensions rather than by centrifuging and ressponding, since in our hands, Novikoff cells showed a lag of approximately one-half generation time both in deoxyribonucleic acid (DNA) synthesis and in cell duplication after centrifugation into a pellet. In subculturing stocks or setting up experiments, all media were prewarmed to 37°C to avoid thermal shock to the cells.

Cloning of the N1-S1 line was easily accomplished by removing microdrops of a dilute (100 cells/ml) suspension to the floors of dry T-30 flasks with the end of a sterile stirring rod. Rapid microscopic examination at 100 × allowed visual selection of those flask containing 1 cell/drop, after which these were flooded quickly with 5 ml of medium and allowed to incubate for 5—7 days. The single nonadhering spherical colony of cells was then separated from the medium, trypsinized briefly with 0.1 ml of 0.05% trypsin and dispersed in fresh medium in the same flask. Within several days, the population was dense enough to be redispersed with trypsin and subcultured to shake flasks.

Assays for thymine and uracil catabolism were accomplished by incubating aliquots of logarithmic-phase cultures with thymine-2,14C or uracil-2,14C for 5 hr (½ generation time) in rubber-stoppered flasks. Cultures were killed and any 14CO2 trapped by injecting sufficient 6 M NaOH through the stoppers to achieve a final NaOH concentration of 1.0 M and then incubating again for at least 5 hr. Aliquots of the flask contents were assayed by acidifying in 50 ml center-well Erlenmeyer flasks containing pellets of NaOH in the center wells, and precipitating and counting the trapped CO2 as barium carbonate.

Amethopterin experiments were conducted as described by Rueckert and Mueller (36). In all cases, in addition to the stated concentrations of amethopterin, the following compounds were also added: adenosine, 5 × 10^-4 M; glycine, 10^-4 M; and serine, 10^-4 M.

The trypsin used was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio (2 × crystalline, 50% MgSO4); amethopterin was obtained from Lederle Laboratories, Pearl River, New York; pyrimidine bases and nucleosides were obtained from the California Corporation for Biochemical Research, except for 5-fluorouracil (5-FU), and 5-fluoro-2'-deoxyuridine, (FUdR) both of which were gifts from Dr. Charles Heidelberger.

RESULTS

Evolution of N1-S1 strain.—As received from Dr. Swim in August, 1960, the N1-S1 strain comprised a heterogeneous population of rounded fibroblasts with a generation time of approximately 18 hr. Although carried in suspension culture, it retained the ability to attach to glass, whence the fibroblast nature of the cells was readily apparent. Clumping of the cells occurred regularly, and it was necessary on every 2nd or 3rd subculture to re-disperse the cells with trypsin or by allowing clumps to settle and subculturing the resulting single cell suspensions. Inoculation of the cells into rats gave excellent takes of tumors over the range of 50 × 10^6 to 50 × 10^8 cells/rat; tumors arose in periods of 5—12 days, depending on the size of the inoculum. In gross morphology, histology, and enzymology, these tumors resembled closely the parent tumor (H. C. Pitot and H. E. Swim, unpublished data). Unlike the parent tumor, N1-S1, inoculated into Fischer strain rats, also gave typical Novikoff tumors (4 out of 4 animals) in 7—10 days; these tumors were then stable enough to be transplanted through at least 3 generations of animals.

Generation time of the N1-S1 line remained constant at 18 hr until December, 1960, when a shift to 15 hr occurred over a period of several weeks. Further shifts to 12 and 10 hr occurred during the following 4 months, at which rate the cells have continued to grow to date. Personal communication from Dr. H. E. Swim indicates that a sister line of N1-S1 carried by him at Western Reserve University since September, 1960, has undergone similar changes in growth rate over the same period of time. At the time of decrease to a 10-hr generation time, cell clumping ceased to be a problem and the ability of the cells to adhere to a glass surface decreased markedly. During the entire period of adjustment, however, no apparent changes occurred in cell morphology or the ability to give typical Novikoff tumors on reinoculation into rats, although some decrease in the rate of "takes" at low inoculation levels was observed. Cloning efficiency of the cells remained at or near 100% throughout. Typical yields of cells in medium 48 (8-77 + 20% horse serum) averaged 3 × 10^4 cells/ml at maximum growth.

Following the last decrease in generation time, the routine culture medium was changed to include 20% beef serum instead of horse serum, with no apparent effect. Reductions in serum content to 10% and 5% also had no effect on generation time, although at the lower concentrations, the yield of cells at maximum growth decreased to
about 1.2 to 1.4 $\times 10^6$ cells/ml. This latter medium, designated No. 71, and containing 5% normal beef serum, has been used for all N1-S1 cultures for the past 18 months. No changes in morphology, growth rate, biochemistry, animal tumors produced, or cloning efficiency of the N1-S1 line have been observed for more than 2 years (360 transfer generations).

Cloning of N1-S1 has given rise to several strains of cells which differ in varying degrees from the parent line with respect to initial colony morphology, growth rate (usually slower), and the ratio of solid to ascitic material produced on reinoculation into rats. However, with few exceptions, all of these "pure" strains have, after variable periods of time, reverted to morphologically mixed cultures with generation times of 10 hr, identical to the parent culture. Multiple clonings have generally had little effect on the final outcome, thus supporting the idea that a fairly high rate of mutation occurs in the N1 line. It is unfortunate that the parent N1-S1 line is no longer capable of attaching to a solid substrate, since this eliminates the possibility of measuring mutation rates directly with any degree of facility.

One series of 2 serial clonings led, however, to the production of an apparently stable subline at each step, both having the same characteristic colony morphology, but different generation times. These sublines, designated N1-S4 and N1-S4-II, have generation times of 11 and 25 hr, respectively, (Chart 1), and when single cells are cultured undisturbed in stationary flasks, round blastula-like colonies are produced from each. Animal tumors produced from both of these sublines appear to grow as rapidly as those derived from the N1-S1 line, and are indistinguishable morphologically from the parent animal tumor. Both sublines have retained stable characteristics for at least 4 months (48 transfer generations) and have been used in several of the experiments described in this and succeeding papers (6, 7).

Before beginning a study of pyrimidine metabolism in the N1-S1 line, it was shown that in these cells, as in the animal tumor, no reductive degradation of thymine or uracil occurs (29, 32). In a typical experiment, flasks containing 28.7 $\times 10^6$ cells suspended in 50 ml of medium were incubated with thymine-$2^14$C, 8.0 $\mu$Ci and 3.30 $\mu$moles...
(2 flasks) and uracil-2-14C, 10.0 μC and 0.44 μmoles (2 flasks). Pairs of control flasks contained only medium plus tracers. All flasks were placed in a shaking incubator at 37°C for \( \frac{1}{4} \) generation time (5 hr), after which 10 ml of 6.0 M NaOH were injected through the stoppers to kill the cells and trap free CO₂. After incubation of these flasks for an additional 5 hr to insure the quantitative reaction of all CO₂, 2.5 ml aliquots were removed from each flask for assay. No acid-labile, barium hydroxide-precipitable radioactivity was recovered with either tracer, indicating a lack of at least one of the steps necessary for reductive pyrimidine catabolism. In other experiments at this institution (6, 7), as well as in work reported by Ives et al. (15), it has also been found possible to account quantitatively for all of the labeled thymine, deoxythymidine, or uracil compounds added to cultures or cell ultrafiltrates in terms of the added precursor or the several anabolic products formed. The lack of pyrimidine catabolism thus greatly simplifies the task of studying anabolic pyrimidine pathways in these cells.

Response to exogenous deoxythymidine.—The presence of high levels of deoxythymidine, (Tdr), in the culture medium has been shown to be inhibitory to a number of cell lines (8, 16, 19, 20), presumably because of inhibition by deoxythymidine triphosphate (TTP) of the reductive synthesis of cytosine deoxyribotides from the corresponding ribotides (21, 33). Because of this, and also due to the fact that the N1-S1 line has a high level of Tdr kinase in the absence of exogenous Tdr (15), it was of interest to determine the effects of various levels of exogenous Tdr on its growth rate.

The data in Chart 2A indicate that the presence of Tdr at concentrations of from \( 10^{-7} \) M to \( 10^{-4} \) M has little effect on growth rate, whereas above \( 10^{-3} \) M, growth is rapidly inhibited, ceasing completely at \( 10^{-2} \) M Tdr. This pattern was repeated in the 2 cloned sublines N1-S4 and N1-S4-II (Chart 2B). This effect was completely reversed by either concomitant or delayed (at least up to 4 generation times) addition of deoxycytidine (Cdr) (Chart 3), complete reversal occurring at a concentration of \( 10^{-4} \) M, in agreement with data obtained by Morris and Fischer for a murine neoplastic cell line (19).

Response to FUDr.—Heidelberger et al. (1, 9, 10, 11), as well as Cohen et al. (2), have demonstrated that inhibition of mammalian cell growth by 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FUDr) occurs primarily via conversion of the compounds to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), and subsequent competitive inhibition by FdUMP of the deoxymyridylate (TMP) synthetase pathway, ultimately blocking DNA synthesis. The inhibition of growth may be reversed by providing an exogenous source of Tdr to circumvent the need for de novo TMP production, or less readily, by high levels of deoxyuridine (Udr), which on conversion to deoxyuridine monophosphate (dUMP) will competitively reverse the inhibition (1).

Because of the high level of Tdr kinase possessed by the N1-S1 line, and because this enzyme is also presumably responsible for the phosphorylation of FUDr to FdUMP, it was of interest to assay the response to this antimetabolite. Chart 4A demonstrates the sensitivity of the N1-S1 line to low levels of FUDr; \( 10^{-4} \) M was sufficient to cause complete inhibition of growth. The lower toxicity of 5-FU (Chart 4B) confirms in part the data of DeVerdier.
and Potter (4, 43), which showed a very low level of nucleoside deoxyribosyl transferase present in Novikoff tumors.

Reversal of FUdr toxicity by Tdr and Udr (Chart 5) followed the expected pattern, with Udr being several orders of magnitude less effective. Since the activity of Udr kinase in N1-S1 is known to be of the same order of magnitude as Tdr kinase, the degree of FUdr toxicity reversal by Udr in N1-S1 is in agreement with the data of Hartmann and Heidelberger for Ehrlich ascites cells (10), which showed the ratio $K_m$ (dUMP):$K_m$ (FdUMP) for TMP synthetase to be about $2 \times 10^6$.

In some of the early toxicity experiments there were indications that a small percentage of the cells was surviving and/or growing for long time periods in $10^{-7}$ M or $10^{-8}$ M FUdr, and in $10^{-3}$ M Tdr, indicating that mutants with altered Tdr kinase might have been present in the culture. Hence, an attempt was made to isolate both FUdr- and Tdr-resistant strains by culturing long-term survivors of minimally toxic concentrations in increasing levels of these compounds. The 2 strains which resulted, designated N1-S1/FUdr and N1-S1/T, were completely resistant to $10^{-4}$ M FUdr and $5 \times 10^{-3}$ M Tdr (Chart 6), respectively. N1-S1/FUdr was cross-resistant to at least $10^{-3}$ M Tdr, was incapable of being rescued from amethopterin poisoning by $10^{-4}$ M Tdr, and, when assayed by the method of Ives et al. (15), showed no measurable level of Tdr kinase. By contrast, N1-S1/T, although insensitive to high levels of Tdr, retained unaltered sensitivity to FUdr, and could still be rescued from amethopterin poisoning by Tdr (Chart 6C). Similar results were obtained with another Tdr-resistant strain, N1-S1-II/T, which was derived from cloned line N1-S1-II. Both N1-S1/T and N1-S1/FUdr strains were tested after having grown for at least 5 generation times in the absence of either inhibitor, and the direct assay of Tdr kinase in N1-S1/FUdr was still negative after 1 month of serial passage in medium lacking FUdr.

R. Gebert, unpublished results. The $K_m$ for Udr is, however, much higher than the $K_m$ for Tdr.

Response to Amethopterin.—As an alternative to the use of FUdr for producing a thymineless condition, the antifolic amethopterin was used in the system of Rueckert and Mueller to block the TMP synthetase pathway (36). In Chart 7, the response of N1-S1 grown in medium No. 71 supplemented with adenosine, serine, glycine, and amethopterin is shown with and without added Tdr. It may be seen that growth of the cells was completely inhibited even at amethopterin concentrations 25-fold less than was used for HeLa (36) and S-180 (8), whereas the presence of Tdr at $10^{-4}$ M was sufficient to allow normal growth. Additional evidence for the lack of appreciable levels of thymine nucleoside deoxyribosyl transferase is also seen in Chart 7, where thymine at $2 \times 10^{-4}$ failed to reverse the effects of amethopterin. This result with whole cells is of interest, since Zimmerman and Seidenberg (43) found thymidine phosphorylase activity in extracts of Novikoff hepatoma cells.

It was hoped that N1-S1 might prove amenable to synchronization by one of the many methods which have been tried for other cell lines (22, 35, 37). Although
CHART 6A.—Comparison of growth of N1-S1 and N1-S1/FUdr on medium No. 71 with and without added FUdr: ● N1-S1 control without FUdr; ○ N1-S1 plus $10^{-4}$ M FUdr; □ N1-S1/FUdr plus $10^{-4}$ M FUdr, continuous culture from N1-S1/FUdr stock; □ N1-S1/FUdr removed from FUdr-containing medium for 43 days and then challenged with $10^{-4}$ M FUdr.

CHART 6B.—Growth curves of N1-S1/FUdr in medium No. 71 without added FUdr: ● control; ○ plus $10^{-4}$ M thymidine (Tdr); □ plus $10^{-4}$ M Tdr and $1.1 \times 10^{-4}$ M amethopterin, supplemented with adenosine, serine, and glycine.

CHART 6C.—Growth curves of N1-S1/T in the presence of $10^{-4}$ M thymidine (Tdr) ○; $1.1 \times 10^{-4}$ M amethopterin (plus adenosine, serine, and glycine); ●; $1.1 \times 10^{-4}$ M amethopterin (plus adenosine, serine, and glycine), plus $10^{-4}$ M Tdr ○; and $10^{-7}$ M 5-fluoro-2'deoxyuridine (FUdr) □.

CHART 7.—Survival of N1-S1 in medium 71 plus amethopterin, supplemented with adenosine, serine, and glycine as described in the text: ● $1.1 \times 10^{-4}$ M amethopterin; ○ $4.4 \times 10^{-4}$ M amethopterin; ○ $1.1 \times 10^{-4}$ M amethopterin plus $10^{-4}$ M thymidine; ○ $1.1 \times 10^{-4}$ M amethopterin plus $2 \times 10^{-4}$ M thymine.

CHART 8.—Survival of several Novikoff cell lines at lowered temperature. ○ N1-S1; ● N1-S3; □ N1-S4. Cells were kept in medium No. 71 in a cold room at temperatures of 3°-6°C. Curves 1, 2, and 3 are viable cell counts (cells not staining with trypan blue). Recovery and growth of cells subcultured to fresh medium at 37°C at various time intervals is shown at A, B, and C.

several different approaches, including cold shock at various temperatures, nutritional shock, reversal of amethopterin toxicity with Tdr, and reversal of Tdr toxicity with Cdr were tried, none gave more than a few percent synchrony, and the attempt was abandoned reluctantly. A useful product of the cold shock experiments, however, was the finding that several of the N1 lines could be maintained at 4°C for several weeks with little loss of viability. Similar results were described by Swim for a number of stationary cell lines (39). The technic evolved consists of subculturing the cells to between 5 and $10 \times 10^4$ cells/ml, allowing growth to occur at 37°C for
DISCUSSION

A number of investigators have observed the loss or diminution of malignant potential of cell strains originating from a variety of tumors (3, 13, 24, 25), while others have reported little or no change (12, 17). Working with the Novikoff hepatoma, Hsu and Klatt (14) reported loss of malignancy in several otherwise apparently stable cell lines. Hotchin (12) was able to obtain tumors from Novikoff tissue culture cells, but the work was carried on over a period of only 8 weeks. The latter strain of cells differed considerably from the ones reported in this paper in that they were more fastidious with respect to both nutrition and pH, and apparently had not undergone the stages of transition (38) from a primary-type culture to an established cell line. In most instances of loss of malignant potential, the available data are insufficient to determine the cause with certainty, although overgrowth of contaminating normal cells from the original explant, mutation and selection of nonmalignant cells or cells unable to survive in the nutritional environment of a host, altered antigenic profile of the cultured cells, and altered karyotype have been variously implicated (13, 38). In relation to the selection of mutants, the selective properties of the various media employed must, of course, be considered.

Although the heterogeneous morphology of the parent cell line together with the results obtained from cloning and adaptation to FUdr and Tdr (Chart 9), all tend to indicate a high rate of mutation in the N1-S1 line, it is apparent either that the preponderance of mutants differ little in those characteristics by which the resulting animal tumor would be defined as being "Novikoff", or, what is more likely, that the stem Novikoff cell line, under the described culture conditions, retains a selective growth advantage over all possible variants.

Since the cell of origin of the Novikoff hepatoma is indeterminate, the question of dedifferentiation of the tumor either in vivo or in cell culture remains open. However, the marked and consistent similarity between animal tumors produced from cultured cells and from the parent tumor would indicate a minimal degree of dedifferentiation attributable to the tissue culture system. That some changes have occurred is indicated by the shorter generation time of the stable cell line, quantitative (but not qualitative) changes in part of the enzyme profile of the

CHART 9.—A diagramatic representation of the several cell lines derived from the Novikoff hepatoma. The stem cell line is in the center, cloned lines are to the left, and mutants to the right of center. All lines produced typical Novikoff tumors in test animals, except N1-S1/T, for which no data are available. Generation times of all lines are 10-11 hr, except N1-S4-II, which is 25 hr. Mutants resistant to 10^{-4} M 5-fluoro-2'-deoxyuridine (FUdr) (N1-S1/FUdr I and II) differ in that I shows an appreciable rate of back mutation while II shows a very low rate.

cells (H. C. Pitot and H. E. Swim, unpublished data) (29), and the ability of the cultured cells to produce tumors in Fischer rats. The latter finding may indicate a change in some of the antigenic properties of the cells. The loss of both cohesiveness and ability to attach to a solid substrate are further indications of altered surface properties in these cells.

The inhibition of N1-S1 growth by high exogenous levels of Tdr is consistent with the high levels of Tdr and TMP kinases present in the wild-type cells (15). Morris and Fischer have demonstrated a similar Tdr kinase dependent inhibition in several murine cell lines (19), and Reichard et al. have shown TTP to be the compound exhibiting negative feedback on the reductive synthesis of dCDP in chick embryo extracts (33). It is interesting to note that, when a Cdr-deficient state is imposed on N1-S1 cells for a period of several generation times (Chart 3B), the culture goes into an exponential death phase, but the survivors are apparently subject to immediate rescue by addition of Cdr. This contrasts sharply with the Tdr-deficient state in HeLa cells, for which Rueckert and Mueller have reported that deprivation of de novo TMP by amethopterin for approximately 1 generation time results in an irreversible thymineless death (35).

Sensitivity to FUdr and the relative lack of sensitivity to 5-FU follow the expected pattern for cells possessing high Tdr kinase and low nucleoside-deoxyribosyl transferase, if the primary site of inhibition is at the TMP synthetase step, as described by Cohen (2) and by Heidelberger et al. (1, 10). That this is probably true is indicated by the ready reversal of FUdr toxicity by Tdr and the less ready reversal by Udr, and by the cross-resistance of N1-S1/FUdr to 5-FU. Resistance to FUdr of the N1-S1/FUdr subline has apparently come about through selection of a mutant lacking Tdr kinase, as evidenced by the demonstrable lack of Tdr kinase in cell supernatant
fractions, the inability of Tdr to rescue amethopterin poisoned cells, and the insensitivity to high exogenous levels of Tdr. Identical results have been obtained with Ni-S1/FUdr cells removed from FUdr for as few as 6 or as many as 120 generation times, indicating that this is truly a mutant cell line, rather than an adaptive response to high FUdr levels. Further support comes from preliminary results obtained with a recently derived strain of N1 cells which attach well to glass and which are also sensitive to FUdr at $10^{-4}$ M. When challenged with $10^{-4}$ M FUdr, most of these cells die, but approximately 1 in $10^8$ survives to establish fully resistant, rapidly growing colonies. These resistant cells are also incapable of surviving in amethopterin medium in the presence of added Tdr, and show no measurable rate of back mutation in the amethopterin plus Tdr system.

In contrast, at least 1 of the Tdr resistant lines, N1-S1/T, retains a very high level of Tdr kinase. Early experiments with this line in which washed cells were assayed for resistance to FUdr led to the erroneous conclusion that the cells were unaffected by this antimetabolite, while, in fact, the endogenous pools of thymidine compounds resulting from previous growth in $10^{-4}$ M Tdr were sufficient to override the presence of $10^{-4}$ M FUdr for the duration of a normal growth curve. Growth of the cells in a Tdr-free medium for several generations before experiments were attempted with FUdr or amethopterin quickly revealed the presence of the kinase, and demonstrated that these cells can grow normally in the face of very high levels of phosphorylated thymidine compounds (6). Although the mechanism of this resistance is unknown as yet, alterations in TMP-kinase activity or in the structure of CDP-dCDP reductase to render it in some way less sensitive to negative feedback by TTP could explain the effect.

The N1-S1 and N1-S1/FUdr cell lines have recently found application in 2 related phases of experimental cancer chemotherapy. Preliminary experiments in conjunction with Dr. Charles Heidelberger of this institute have been performed using the wild type cells plus the mutant cell system as a model for human tumors susceptible and resistant to the effects of fluorinated pyrimidines. This system has been used to test the efficacy of new derivatives of this class of compounds in inhibiting the growth of resistant tumors, in the hope of circumventing the need for Tdr kinase in the final activation of the tumor-inhibitory compound. In a related series of experiments, to be reported elsewhere, Dr. William Wolberg of the University of Wisconsin Cancer Research Hospital has used the same pair of cell types as a model in determining by autoradiography the responses of a number of human tumors to the administration of 5-FU and FUdr. Thus far, excellent correlations have been obtained between the clinical responses of tumors to chemotherapy and the autoradiographic assay of uptake of several labeled pyrimidine compounds by biopsy specimens in the presence or absence of the drugs, as compared to the expected response and uptake in the 2 lines of tissue culture cells.

Since a considerable body of metabolic information has been accumulated for the Novikoff hepatoma in vivo, it is indeed unfortunate that the cell of origin of this tumor cannot be defined with certainty, in order that normal versus malignant tissue comparisons could be made. This is particularly true now that stable Novikoff cell cultures are available in cloned and mutant forms. This does not detract from the fact, however, that ease of growth, long-term stability, high cloning efficiency, and ability to produce typical animal tumors combine to make these cell lines extremely useful as basic biochemical tools. In addition, the lack of apparent qualitative dedifferentiation in the cultured cells allows valid in vivo versus in vitro comparative studies.

Other papers of this series (6, 7) report the results of a number of studies on the anabolism of pyrimidine nucleosides and deoxynucleosides using several of the N1 cell lines. The studies were facilitated by the high nucleoside-kinase activities in these cells, and by the complete lack, as in the tumor in vivo, of any catabolism of thymine or uracil.

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Pyrimidine Metabolism in Tissue Culture Cells Derived from Rat Hepatomas: I. Suspension Cell Cultures Derived from the Novikoff Hepatoma

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